EUROPEAN COMMISSION
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JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Verification of Performances of 1507 and 59122 Event-specific Methods on the Hybrid 1507x59122 Using Real-time PCR

Validation Report

Biotechnology & GMOs Unit
Institute for Health and Consumer Protection
DG Joint Research Centre

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Executive Summary

The JRC as Community Reference Laboratory (CRL) for the GM Food and Feed (see Regulation EC 1829/2003), has carried out an in-house verification study to assess the performance of two quantitative, event-specific methods, previously validated on the parental lines, to detect and quantify the DAS-Ø15Ø7-1 (here after referred to as 1507 maize) and the DAS-59122-7 (here after referred to as 59122 maize) transformation events on flour from the hybrid maize line DAS-Ø15Ø7-1xDAS-59122-7 (here after referred to as 1507x59122 maize) combining the two thereof traits (unique identifier DAS-Ø15Ø7-1xDAS-59122-7). The study was conducted according to internationally accepted guidelines.

Dow AgroSciences provided the method-specific samples (ground maize flour of hybrid 1507x59122 and null), whereas the JRC prepared the verification samples (calibration samples and blind samples at unknown GM percentage).

The results of the in-house verification study were evaluated with reference to ENGL method performance requirements (http://gmo-crl.jrc.it/doc/Method%20requirements.pdf) and the validation results for the two parental lines (http://gmo-crl.jrc.it/statusofdoss.htm).

The results of in-house verification are publicly available under http://gmo-crl.jrc.it/.

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Document Approval		
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1. Introduction

The Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute of Health and Consumer Protection) as Community Reference Laboratory for the GM Food and Feed (see Regulation EC 1829/2003) carried out an in-house verification of the event-specific methods for the detection and quantification of 1507 and 59122 in the hybrid maize line combining the two traits derived through traditional breeding techniques between progeny of 1507 and 59122 maize. The single methods had been previously validated further to collaborative trial on the single parental lines (http://gmo-crl.jrc.it/statusofdoss.htm).

Upon reception of methods, samples and related data, the JRC carried out the scientific evaluation of documentation and the in-house testing of the methods, according to the requirements of Regulation (EC) 641/2004 and following its operational procedures.
The CRL method verification was carried out in December 2005-March 2006.

Genomic DNA from wild type maize and from the maize line 1507x59122 was extracted following the methods enclosed in the validated protocols for events 1507 and 59122 respectively (http://gmo-crl.jrc.it/).

The operational procedure of the in-house verification comprised the following module:

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology consists of two event-specific real-time quantitative TaqMan® PCR procedures for the determination of the relative content of event 1507 and 59122 DNA to total maize DNA from the hybrid line. The 1507 and 59122 events were quantified with reference to a maize endogenous system obtained from an *hmg* gene (high mobility group). The procedure is a simplex system.

The PCR assay has been optimised for use in real-time PCR instruments for plastic reaction vessels.

The study was carried out in accordance with the following internationally accepted guidelines:

- ✓ ISO 5725 (1994).
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995).

2. Materials

For the validation of the quantitative event-specific method, the 1507x59122 genomic DNA was extracted from ground maize flour, lot HW03122117HWG11RW300, while the control DNA was extracted from a non-GM maize line (Lot PIP20LBN).

Samples containing mixtures of 0% and 100% 1507x59122 maize genomic DNA at different GMO concentrations were prepared by the JRC.

The protocols (reagents, concentrations, primer/probe sequences, amplification profile) used in the in-house verification are those already published as validated methods for the 1507 and for 59122 event.

Table 1 shows the GM levels of unknown samples used in the verification of the 1507 and 59122 methods on the hybrid DNA of 1507x59122.

Table 1. GM contents in the unknown samples

1507 GM % (GM copy number/maize genome copy number *100)	**59122 GM %** (GM copy number/maize genome copy number *100)
0.10	0.10
0.50	0.40
0.90	0.90
2.00	2.00
5.00	4.50

3. Experimental design

Eight runs for each method were carried out. In each run, samples were analyzed in parallel with both the GM-specific system and the reference system. Five GM-levels were examined per run (from 5.00% down to 0.10% for 1507 and from 4.5% down to 0.10% for 59122) in two replicate samples. Each sample was analyzed in triplicate. On the whole, for each method (1507 and 59122), quantification of the five GM levels was performed as an average of sixteen replicate samples/GM-level, each resulting from an average of three repetitions. An Excel spreadsheet was used for the calculation of the GM%.

4. Methods

4.1 Description of the operational steps

For specific detection of event 1507 genomic DNA, a 58-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For specific detection of event 59122 genomic DNA, an 86-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For relative quantification of event 1507 and 59122 DNA, a maize-specific reference system amplifies a 79-bp fragment of *Hmg* (high mobility group), a maize endogenous gene, using a pair of *hmg* gene-specific primers and an *hmg* gene-specific probe labelled with FAM and TAMRA.

The standard curves are generated for the *hmg* and 1507 and 59122 systems respectively, by plotting the Ct-values measured for the calibration points against the logarithm of the DNA copy numbers, and by fitting a linear regression line into these data.
Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves.

For the determination of the amount of 1507 (or 59122) DNA in the unknown sample, the 1507 (or 59122) copy number is divided by the copy number of the maize reference gene *hmg* and multiplied by 100 to obtain the percentage value (GM% = GM-specific system/maize reference system * 100).

For detailed information on the preparation of standard curve calibration samples refer to the protocols of validated methods under http://gmo-crl.jrc.it/summaries/TC1507-report_mm.pdf and http://gmo-crl.jrc.it/summaries/59122_val_report.pdf.

5. Deviations reported

No deviations from the protocols of the two validated methods were reported

6. Summary of results

6.1. PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula ((10^(-1/slope))-1)*100] of the standard curves and of the R^2 (expressing the linearity of the regression) reported for both PCR systems in the eight runs, are summarised in Table 2 and 3.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 1507 method on hybrid 1507x59122

Run	1507			*Hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.48	93.66	0.97	-3.59	89.83	0.98
2	-3.39	97.24	0.99	-3.52	92.35	0.99
3	-3.46	94.41	0.98	-3.61	89.25	0.99
4	-3.28	98.10	0.99	-3.60	89.57	0.99
5	-3.30	99.12	0.99	-3.58	90.25	0.98
6	-3.46	94.46	0.99	-3.53	91.92	0.99
7	-3.35	98.72	0.99	-3.58	90.39	0.99
8	-3.40	96.71	0.99	-3.50	93.25	0.99
Mean	**-3.39**	**96.55**	**0.98**	**-3.56**	**90.85**	**0.99**

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 59122 method on hybrid 1507x59122

Run	59122			*Hmg*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.45	95.03	1.00	-3.41	96.62	1.00
2	-3.22	95.50	1.00	-3.25	96.92	0.99
3	-3.40	96.93	1.00	-3.12	90.94	0.99
4	-3.55	91.24	1.00	-3.50	93.10	1.00
5	-3.28	98.32	0.99	-3.33	99.57	1.00
6	-3.40	96.85	1.00	-3.50	93.21	0.99
7	-3.27	97.71	1.00	-3.36	98.61	1.00
8	-3.40	96.81	0.99	-3.26	97.26	1.00
Mean	**-3.37**	**96.05**	**0.99**	**-3.34**	**95.78**	**1.00**

Data reported in Table 2 and 3 confirm the good performance characteristics of the tested methods. In fact, the R^2 value of the regression line for the 1507 method is above 0.98; similarly the R^2 value of the regression line for the 59122 system is greater than 0.99. PCR efficiencies are higher than 90%.

7. Method performance requirements

The results of the in-house verification are reported in Table 4 for the 1507 and the 59122 methods. These are evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by CRL.

In table 4 estimates of both accuracy and precision for each GM level and for both methods are reported.

Table 4. Estimates of accuracy and precision for the 1507 and 59122 methods on maize 1507x59122

1507					
Unknown sample GM%	**Expected value (GMO %)**				
	0.10	**0.50**	**0.90**	**2.00**	**5.00**
Mean	**0.11**	**0.45**	**0.88**	**1.80**	**4.84**
SD	0.023	0.041	0.087	0.126	0.477
RSDr%	21.25	9.04	9.96	7.00	9.86
Bias%	6.30	-9.71	-2.64	-10.15	-3.24
59122					
Unknown sample GM%	**Expected value (GMO %)**				
	0.10	**0.40**	**0.90**	**2.00**	**4.50**
Mean	**0.10**	**0.45**	**0.93**	**2.10**	**4.10**
SD	0.015	0.064	0.160	0.323	0.719
RSDr%	15.17	14.10	17.25	15.35	17.52
Bias%	0.56	12.76	2.90	5.12	-8.79

According to the ENGL acceptance criteria, the accuracy of the quantification, measured as bias from the accepted value, should be within 25% over the whole dynamic range, and the relative repeatability standard deviation, which measures the intra-laboratory variability, should lie within 25% at each GM level.

As shown in Table 4, it can be observed that the accuracy of the 1507 method is acceptable over the whole dynamic range. The relative repeatability standard deviation (RSDr) is well within the limits set by the acceptance criteria.

Similarly, the RSDr of the 59122 method is definitely acceptable at all tested GM concentrations. The accuracy, expressed as bias, maintains below the 25% at each GM concentration showing a maximum (12.76%) at the 0.40% level.

Therefore, the two methods fully satisfy the acceptance criteria for CRL verification of GMO detection and quantification methods previously validated through collaborative trial on the parental maize lines.

8. Comparison of method performance between hybrid and parental lines

A synoptic comparison of the two method performances in the hybrid maize and parental lines respectively is shown in Table 5 and 6.

Table 5. Comparison of accuracy and precision of 1507 method in the hybrid and parental line

Accuracy and precision of 1507 quantification in hybrid 1507x59122			**Accuracy and precision of 1507 quantification in parental line 1507***		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
			0.00	0.00	0.00
0.10	6.30	21.25	**0.10**	6.00	18.11
0.50	-9.71	9.04	**0.50**	-4.00	11.70
0.90	-2.64	9.96	**0.90**	3.70	7.68
2.00	-10.15	7.00	**2.00**	-1.70	8.48
5.00	-3.24	9.86	**5.00**	8.40	14.41

***method validated (http://gmo-crl.jrc.it/statusofdoss.htm)**

Table 6. Comparison of accuracy and precision of 59122 method in the hybrid and parental line

Accuracy and precision of 59122 quantification in hybrid 1507x59122			Accuracy and precision of 59122 quantification in parental line 59122*		
GM%	**Bias (%)**	**RSDr (%)**	**GM%**	**Bias (%)**	**RSDr (%)**
0.10	0.56	15.17	**0.10**	29	18.16
0.40	12.76	14.10	**0.40**	15	13.89
0.90	2.90	17.25	**0.90**	9	15.84
2.00	5.12	15.35	**2.00**	7	13.59
4.50	-8.79	17.52	**4.50**	-1	8.45

***method validated (http://gmo-crl.jrc.it/statusofdoss.htm)**

The 1507 method showed similar performance characteristics on the hybrid product as on the parental line with the exception of a higher level of bias at the 2.00% level (-10.15) and at the 0.5% level (-9.71); both values are however well within the performance requirement for the accuracy (maximum 25% of bias) set by ENGL.

The 59122 method shows better performances in the hybrid line as compared to the performances displayed in the parental line in terms of accuracy and similar values of repeatability.

Therefore, the in-house method verification has demonstrated that the 1507 and the 59122 methods can be equally applied in quantification of the respective events in the hybrid maize product.

9. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (available under http://gmo-crl.jrc.it). The method acceptance criteria were reported by the applicant and used to evaluate the method prior the in-house verification.

The results obtained during the present study indicate that the methods validated on the parental GM lines show a comparable performance when applied to the material combining the two traits.

10. References

Horwitz, W. (1995) Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, **67**, 331-343.

International Standard (ISO) 5725. 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Swizerland.